A CHEMICAL REAGENT FOR THIAMINE

DISSERTATION

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A CHEMICAL REAGENT FOR THIAMINE*

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The rapid advances in the study of the physiological rôle of thiamine (vitamin B_1) make its direct quantitative estimation highly desirable. Few chemical tests have been proposed which offer the prospect of application to solutions or concentrates for this purpose.

Jendrassik (1) while working in our laboratory tried to find a specific chemical reagent which would produce a qualitative color reaction with the antineuritic vitamin. He found that solutions containing this vitamin invariably reduced ferric ferricyanide to form blue-colored solutions, which he assumed owed their color to the presence of Prussian blue (ferric ferrocyanide). Levine (2) made a critical study of Jendrassik's reaction and found that this test was given by ortho- and polyphenols. Nevertheless the use of ferricyanide may have suggested a line of thought to Barger (3), who oxidized the vitamin with alkaline ferricyanide to form a pale yellow-blue fluorescent compound now known as thiochrome. The thiochrome test has been made the basis of a method for the quantitative estimation of thiamine by Jansen (4), who used the fluorometer apparatus of Cohen (5) for measuring the fluorescence of the thiocrome after it had been extracted with isobutyl alcohol. Certain substances interfere with this reaction, since complete recovery of thiamine added to urine cannot be obtained.

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The method also involves the use of special apparatus and the results obtained vary as much as 20 per cent from those obtained by the bradycardia method of Birch and Harris (6).

Spruyt (7) proposed the use of phosphotungstic acid to precipitate thiamine from extracts of foodstuffs and estimated the centrifuged precipitate by means of hematocrit tubes. Rosenthaler (8) reported the use of Reinecke's salt as well as potassium tetranitritodiamino cobaltate for precipitating the vitamin. However, these tests are not specific. Naiman (9) reported the use of a solution of bismuth iodide in potassium iodide as a sensitive reagent for the thiazoles. He suggested the possibility of using this reagent as the basis of a gravimetric determination of thiamine. No specificity was claimed and it is well known that the solution reported is classed with the alkaloidal reagents.

A Pauly (10) color reaction for thiamine was first reported by Jansen and Donath (11) who employed diazotized sulfanilic acid as the reagent. Kinnersley and Peters (12) claimed that their yeast fractions did not fractionate with biological activity when they used the Koessler and Hanke modification of this reaction (13). Guha and Drummond (14) also reported that active antineuritic fractions obtained from yeast gave feeble Pauly reactions. Odake (15) claimed that his vitamin B_1 crystals gave a slow, pink diazo reaction. Nevertheless, Tschesche (16) and also van Veen (17) were unable to find any activity of their crystalline thiamine preparations when treated with diazotized sulfanilic acid. Van Veen was of the opinion that a reaction should take place and he was at a loss to explain the inactivity of his preparations with the reagents.

Kinnersley and Peters (18) were of the opinion that fine shades of alkalinity influenced the coupling of the vitamin. When the Koessler and Hanke modification of this reaction was used, a yellow coloration was obtained, whereas solutions more strongly alkaline resulted in pink colors which faded gradually and were always tinged with yellow. Although the formaldehyde modification of Kinnersley and Peters (18) increased the stability of the pink colors obtained with thiamine, the test was unsatisfactory for colorimetric work because of the yellow tinge which persisted. Even though Kinnersley and Peters were not successful in placing

the reaction on a colorimetric basis, their formaldehyde-azo test was a definite advancement toward developing a colorimetric test for thiamine, which minimized the coupling of histamine type compounds with diazotized sulfanilic acid.

Deviatmin (19) modified the Kinnersley-Peters test by heating the reagent with the vitamin test solution before comparing with standards. This modification may prove to be of doubtful value, since diazotized sulfanilic acid decomposes upon heating.

Since Ehrlich (20) found diazotized sulfanilic acid useful as a reagent, many workers have sought to use this compound for coupling with substances of biochemical interest. It was surmised that the investigation of other derivatives of aniline or the naphthylamines might afford a reagent of greater specificity for thiamine than diazotized sulfanilic acid. A diazotized amine which would react with the vitamin to form a colored water-insoluble compound would afford the possibility of separating this entity from extraneous material, and so provide a better basis for a chemical estimation. A reagent which would not react with other biologically important compounds but only with thiamine to produce a highly colored precipitate would be ideal. Our search for such a reagent has, we believe, been successful.

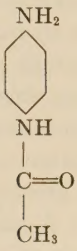
EXPERIMENTAL

A great many amines were diazotized according to the method of Kinnersley and Peters (18), which involved the use of sodium nitrite and a solution of sulfanilic acid in hydrochloric acid. We employed one molecular equivalent of the amine tested in place of sulfanilic acid.

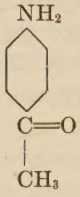
The attempts to couple thiamine with diazotized amines in an acid solution to produce colorations or colored precipitates were unsuccessful. This confirmed the experience of Kinnersley and Peters (18). When solutions of the diazotized amines were made alkaline and added to aqueous test solutions of thiamine, coupling took place, as evidenced by a series of color changes. Similar experiments were carried out simultaneously with test solutions of histidine, since it was most desirable to find a diazotized amine which would differentiate between this compound and the vitamin. The diazotized naphthylamines and the diazotized derivatives of

aniline coupled in an alkaline solution with thiamine to produce a variety of red colors, whereas histidine coupled under similar conditions to produce yellow- and orange-colored solutions.

Of the amines investigated the alkaline diazotates of *p*-aminoacetanilide and *p*-aminoacetophenone produced purple-red-colored precipitates with solutions containing thiamine.



p-Aminoacetanilide



p-Aminoacetophenone

A brief preliminary report of these reagents has been made (21).

To bring about a diazotization reaction with *p*-aminoacetanilide, it was first necessary to dissolve this compound in hydrochloric acid as a preliminary step. Upon standing, acid solutions or dilute aqueous solutions of this amine decompose and assume a deep red color. The decomposition takes place in the dark as well as in the light. Evidently a hydrolysis takes place slowly at the point where the acetyl radical is attached to the secondary amino group in the molecule.

Since *p*-aminoacetophenone is fairly stable in aqueous solutions, our subsequent work was confined to this amine which proved to satisfy the requirements for its use as a reagent for thiamine. In our hands this reagent is best prepared from the following three solutions, freshly mixed before using.

Solution A—3.18 gm. of *p*-aminoacetophenone (Eastman Kodak Company, No. 631) are dissolved in 45 cc. of concentrated hydrochloric acid (37 per cent) and made up to a final volume of 500 cc. in a volumetric flask with distilled water. Precautions are taken to keep the solution in a glass-stoppered flask and to avoid the presence of strong light when not in use. The activity of this solution is unaltered after standing for 6 months.

Solution B—22.5 gm. of C. P. sodium nitrite are dissolved in distilled water and made up to a final volume of 500 cc. in a glass-

stoppered volumetric flask. This reagent begins to deteriorate after standing for a month. It is best preserved at refrigeration temperatures.

Solution C—20 gm. of C. P. sodium hydroxide are dissolved in 600 cc. of distilled water; 28.8 gm. of C. P. sodium bicarbonate are then added and the solution made up to a final volume of 1 liter with distilled water.

Directions for Diazotization—The diazotization reaction is carried out in an ice bath with use of 1 part by volume each of Solution A and of Solution B. This reaction requires a period of about 10 minutes. The mixture is agitated by means of an electric stirrer. At the end of this period 4 parts of Solution B are added to the resultant mixture. The solution is stirred and maintained at a temperature of 0–5° for at least 20 minutes. When the reaction is completed, the diazotized solution remains satisfactory for use at least 12 hours at refrigeration temperatures. It is advisable to prepare fresh solutions daily.[1]

Directions for Making Final Reagent—In making the final reagent for reaction with the vitamin, add 20 cc. of freshly diazotized *p*-aminoacetophenone to a flask containing 275 cc. of Solution C. When these reagents are mixed, a purple coloration appears. This color disappears on stirring with an electric stirrer for a period of 5 to 10 minutes. The test solution must be adjusted to pH 5.0 to 6.0 immediately before it is allowed to react with the final reagent.

For a simple qualitative test on a solution containing thiamine, small Wassermann tubes are well suited for carrying out the reaction. 2 cc. of final reagent were added to 1 cc. of the thiamine test solution. The reaction is characterized by the formation of a purple-red coloration within 20 minutes and upon standing a precipitate of the same color settles out. For solutions containing less than 5 micrograms per cc. it is necessary to use larger quantities of the test solution. With smaller quantities of the vitamin, a small hand-glass may be necessary for observing the colored precipitate which settles out within 20 to 24 hours. By using the hanging drop technique, as little as 0.1 microgram of synthetic thiamine may be detected under the microscope.

The purple-red precipitate is flocculent and exhibits the follow-

[1] It has been found that this reagent retains its activity for a week.

ing properties: it is insoluble in water, concentrated alkali, dilute acids, and petroleum ether; is soluble in 95 per cent ethyl alcohol, propyl, butyl, and isobutyl alcohol, acetone, dioxane, glacial acetic acid, ethyl ether, carbon tetrachloride, chloroform, benzene, toluene, and xylene.

Of the solvents mentioned, isobutyl alcohol, toluene, and xylene were found best for extracting the colored suspension to form a colored non-aqueous liquid phase. Of these latter solvents, the aromatic compounds seemed to possess the best working properties for quantitative work, since they are least soluble in water.

The reagent has been tried successfully with the formation of the characteristic purple-red precipitate on samples of defatted wheat germ, defatted rice polishings, Seidell's international adsorbate, Anheuser-Busch yeast concentrate No. 2, Anheuser-Busch yeast adsorbate, Eli Lilly rice polishings adsorbate No. 928,389, crystalline thiamine (Merck), both natural and synthetic, as well as synthetic thiamine crystals supplied by the Winthrop Chemical Company. It was found that the colored precipitate could be removed from extraneous material and recovered by extraction with acetone and evaporation of this solvent with the subsequent addition of water.

In order to ascertain whether or not the new reagent reacted with other biologically important compounds, it was tested with the following substances: acetic acid, acetone, adrenalin, adenine, albumin, ascorbic acid, betaine, caffeine, cytosine, casein hydrolysate, creatine, creatinine, dextrose, ethyl alcohol, fructose, galactose, gelatin hydrolysate, glutathione, guanidine, guanine, hydroquinone, inositol, insulin, lactose, methyl alcohol, nicotinic acid, nicotinic acid amide, nucleic acid, oxalic acid, phenol, phloroglucinol, quinine, resorcinol, riboflavin, saccharin, sarcosine, sucrose, theelin, thioneine, thiochrome, trimethylamine, thymine, tyramine, uracil, urea, uric acid.

Each of the twenty-two amino acids was also tested. Of all the substances tested, inositol (ash-free, Eastman, No. 1252) was the only compound which produced a colored precipitate with the reagent. The precipitate obtained with this compound was flocculent and greenish blue in color. When this precipitate was collected on filter paper and washed with distilled water, it changed to a yellow color. It was soluble in dilute acids and slightly in

distilled water. The inositol precipitate is insoluble in dilute alkali. In aliphatic solvents it imparted a slight yellow coloration; in aromatic solvents it is insoluble. Inositol did not interfere with the reaction when thiamine was allowed to react with the reagent, since its reaction product could be removed following filtration by washing with dilute acid.

In an attempt to elucidate the mechanism of the reaction involved in the formation of the purple-red precipitate obtained by allowing thiamine to react with the reagent, some of the following intermediate compounds used in the synthesis of thiamine were studied: Merck, 37-AR-84(2), 2-methyl-5-bromomethyl-6-aminopyrimidine hydrobromide; Merck, 37-AR-85(3), 4-methyl-5-β-hydroxyethylthiazole; Merck, 37-AR-86(1), 5-ethoxymethyl-6-aminopyrimidine; Merck, 37-AR-105, 4-methyl-5-β-hydroxyethylthiazole methiodide.

Both pyrimidine derivatives produced a yellow coloration when permitted to react with the final reagent, whereas the thiazoles produced purple-red precipitates whose properties were similar to the colored precipitate obtained with vitamin solutions. It is of interest to note that the free thiazole portion of the molecule reacted much more slowly and produced a smaller amount of precipitate than an equivalent quantity of the quaternary form, which reacted almost instantaneously.

The following synthetic compounds were obtained from Dr. A. R. Todd of the Lister Institute, London, and were tested with the reagents: 4-methylthiazole, 3-(6′-amino-4′-ethylpyrimidyl-5′)-4-methyl-5-β-hydroxyethylthiazolium hydrochloride, 3-(6′-amino-4′-methylpyrimidyl-5′)-4-methylthiazolium chloride hydrochloride, 4-amino-5-aminoethyl-2-methylpyrimidine hydrochloride, 3-(6′-amino-2′-methylpyrimidyl-5′-5′)-methyl-4-methylthiazolium chloride hydrochloride, 3-(6′-hydroxy-2′-methylpyrimidyl-5′)-methyl-4-methylthiazolium chloride hydrochloride.

The only compound in this group which reacted with the reagent to produce a purple-red precipitate was the inactive isomer of thiamine, 3-(6′-amino-4′-ethylpyrimidyl-5′)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochloride. It is of interest to note that this compound was similar in its reactivity to the free thiazole portion of the vitamin which reacted much more slowly with the reagent than the vitamin itself, which contains a methyl-

ene bridge between the thiazole and pyrimidine portions of the molecule.

It was also found that under controlled conditions the colored precipitate, obtained by allowing the reagent to react with thiamine, obeyed Beer's law in aliphatic as well as aromatic solvents.

The absorption spectra[2] of purified samples of colored precipitates prepared with the reagent from Eli Lilly adsorbate (rice polishings), as well as Merck and Winthrop synthetic thiamine, indicated a maximum absorption of 325 mμ for all samples; in the visible region the Winthrop and Merck products exhibited identical maxima at 516 mμ, whereas the precipitate obtained from the Eli Lilly adsorbate exhibited the same type of absorption but with a maximum at 520 mμ. This may have been due to small quantities of impurities present in the product obtained from the adsorbate; nevertheless the results check within the limits of photographic technique and indicate that the reaction products obtained from natural sources are similar to those obtained by reaction of the reagent with the synthetic vitamin.

DISCUSSION

The reagent described herein seems to be specific for the 4-methyl-5-β-hydroxythiazole portion of the thiamine molecule. In applying this reagent for quantitative work, we have had considerable difficulty in obtaining approximately quantitative extraction of the vitamin from such biological materials as yeast, wheat germ, etc. After extraction, the problem of concentrating the vitamin without destruction presented itself. Adsorbates seem best suited for this purpose, since they also allow the removal of extraneous material without destruction of the vitamin. It should be mentioned that adsorbates had to be specially treated so that eluates made therefrom did not carry along inorganic substances which interfered with the activity of the reagent. High salt concentrations and metallic ions were found to influence the effectiveness of the reagent. In working with adsorbates from extracts of natural products it is difficult to obtain 100 per cent elution of the vitamin.

These obstacles must be overcome before a method generally

[2] The authors are indebted to Dr. C. E. Bills and Dr. F. G. McDonald of Mead Johnson and Company for the absorption spectra studies.

applicable can be worked out for using the new reagent as part of a quantitative chemical procedure for the assay of foodstuffs and biological materials. Further work along these lines is in progress in this and other laboratories.

SUMMARY

Two reagents have been described which react with the thiazole portion of thiamine to form purple-red compounds which are highly insoluble in water but soluble in selective organic solvents. Alkaline diazotized *p*-aminoacetophenone was found to be the better of the two reagents reported. Many obstacles, such as complete quantitative extraction and concentration of thiamine from biological materials, must be overcome before the reagent is applicable in quantitative work.

BIBLIOGRAPHY

1. Jendrassik, A., *J. Biol. Chem.*, **57**, 129 (1923).
2. Levine, V. E., *J. Biol. Chem.*, **62**, 157 (1924–25); **64**, 591 (1925).
3. Barger, G., Bergel, F., and Todd, A. R., *Nature*, **136**, 259 (1933).
4. Jansen, B. C. P., *Rec. trav. chim. Pays-Bas*, **55**, 1046 (1936).
5. Cohen, F. H., *Rec. trav. chim. Pays-Bas*, **54**, 133 (1935).
6. Birch, T. W., and Harris, L. J., *Biochem. J.*, **28**, 602 (1934).
7. Spruyt, J., Dissertation, Amsterdam (1933).
8. Rosenthaler, L., *Z. Vitaminforsch.*, **5**, 276 (1936).
9. Naiman, B., *Science*, **85**, 290 (1937).
10. Pauly, H., *Z. physiol. Chem.*, **42**, 508 (1904).
11. Jansen, B. C. P., and Donath, W. F., *Proc. K. Akad. Wetensch. Amsterdam*, **29**, 1390 (1926); *Verslag. Akad. Wetensch. Amsterdam*, **35**, 923 (1926); *Geneesk. Tijdschr. Nederl.-Indië*, **66**, 810 (1927).
12. Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, **21**, 789 (1927); **22**, 422 (1928).
13. Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, **39**, 497 (1919).
14. Guha, B. C., and Drummond, J. C., *Biochem. J.*, **23**, 880 (1929).
15. Odake, S., *Bull. Agric. Chem. Soc. Japan*, **8**, 113 (1932); **10**, 71 (1934).
16. Tschesche, R., *Chem.-Ztg.*, **56**, 166 (1932).
17. van Veen, A. G., *Z. physiol. Chem.*, **208**, 125 (1932).
18. Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, **28**, 1187 (1934).
19. Deviatmin, V. A., *Compt. rend. Acad. sc. U. R. S. S.*, **4**, 67 (1936).
20. Ehrlich, P., *Deutsch. med. Woch.*, **9**, 549 (1883); **10**, 419 (1884).
21. Prebluda, H. J., and McCollum, E. V., *Science*, **84**, 488 (1936).

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